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Separation and Characterization of Keratin Components of Merino Wool. I: A General Consideration on Methodology

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This paper deals with a problem of design for experimental procedures to be applied most appropriately to the disruption and dissolution of Merino wool fiber to prepare the chemically unmodified histological protein components. For the purpose, a number of works hitherto made on separation and characterization of the histological components of wool have been reviewed and assessed critically. As the result, a method using ultrasonication, designated "stepwise disintegration method", is proposed as the ideal, which consists of a series of successive experimental procedures, *i.e.*, removal of cuticular cells, disruption of decuticled fiber into cortical segments and remnant components, and separation of the former into the ortho- and paracortical segment.

I. INTRODUCTION

The central dogma, enunciated by Crick[1] in 1958, allows us to deduce a guiding principle that the higher structure of a given protein molecule is decided by its primary structure. This means, in turn, that the determination of the primary structure has priority over that of the higher structure in the conformational study of proteins. The principle has been proved valid for a number of proteins. However, any approach according to this principle may not be applicable to structural proteins constituting wool fiber. The major reason consists in the fact that wool fiber comprises a variety of histological protein components, whose primary structures must, of course, be different from one another. In fact it has been reported by Corfield *et al.* that a large number of different primary structures were assigned to oligopeptides obtained by controlled degradation of oxidized wool and, subsequently, by tryptic proteolysis.[2] The second reason is, as well known, that most keratin molecules located in the cuticular and cortical cells of wool are linked intermolecularly by disulfide bonds so that they cannot be extracted as single molecule without rupture of such bonds, and, even after brought into solution, they cannot be gained in crystalline form as such in the case of enzymes. Another complexity in the conformational study of wool keratin is that there is no criterion to define absolutely the degree of chemical and other modifications from which a given specimen suffers.

Thus it seems impossible to isolate some major protein components from wool, each being regarded as a chemical entity of wool and having a definite primary structure. For instance, a question aroused is whether or not all the protofibrils in wool

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fiber comprise keratin molecules having the same primary structure, but no answer is available at present. Therefore, one should be satisfied if, under a defined extraction condition and even in repeated runs of the extraction experiment, some protein components could be obtained from wool, each of which gives invariable chain length and amino acid composition (though the sequence is unknown), as well as invariable information on the secondary structure, *e.g.*, the helix content. In addition it is, of course, required that separated proteins are in native conformations as much as possible.

The aim of our research work to be done is to elucidate the higher structure of keratin components constituting the cortical cells of Merino wool. Thus this article deals with a design for the most appropriate procedures to be applied especially to the disruption and dissolution of wool fibers. For the purpose we will review and critically assess a variety of works hitherto made on separation and characterization of protein components of wool.

II. DIRECT EXTRACTION OF KERATIN COMPONENTS AFTER RUPTURE OF DISULFIDE BONDS

It is well known that there are two different methods for rupture of cystine bonds to isolate structural proteins from wool: one is by oxidation and the other by reduction. By using the former method, many investigators have extracted wool protein fractions which differed in their sulfur content. In most cases the oxidized wool was subjected to the classical fractionation procedure, proposed by Alexander *et al.*,[3,4] to obtain the so-called α-, β- and γ-keratoses. Analyses for the fractions have been made intensively. However, it has recently found that an appreciable amount of protein fraction, which showed far higher contents of tyrosine, phenylalanine and others than the average for native wool, was dissolved during extraction of wool with performic acid.[5] This result might imply that the oxidation method yields chemically modified protein fractions, and for this reason, it was decided not to apply this method to our purpose. Therefore, our subsequent review and assessment will be confined to works by the reduction method, which might be chemically milder than the former.

A series of intensive works on extraction by reduction and characterization of keratin components from wool have been made mainly by the staffs of the CSIRO Wool Research Laboratories, Melbourne. Their first important finding was that on rupture of the disulfide bonds by reduction, about 70–80% of the wool could be extracted into solution, and the soluble portion was fractionated into two main groups: one has a sulfur content much higher than that of the parent wool; while the other a sulfur content less than half that of wool itself.[6] Further investigations on fractionation and chemical as well as physicochemical characterization of these soluble portions have been the subject of the Laboratories.[7~11] Thus, before 1965, it was confirmed that the structural unit of wool is described in terms of a multichain structure in which the low-sulfur protein molecules, which form the microfibrils, are embedded in the high-sulfur ones existing as the matrix.[12]

Their subsequent studies have been concentrated largely to the low-sulfur protein extracted from reduced and carboxymethylated wool. Two major fractions designated

Component 7 and 8 were recognized in an electrophoresis experiment on S-carboxymethylated low-sulfur protein, and subjected to various chemical and physicochemical determinations.[13,14] It was found that these fractions were more than 50% helical in aqueous solution and has a molecular weight of ca. 45000 on the basis of their elution volumes in gel-filtration.[15] On the other hand, the total low-sulfur protein fraction was partially proteolyzed with Pronase P by Crewther and Harrap.[16] From the digest an acid-precipitable fraction was yielded, which showed an axial ratio of 8–10: 1. Further it was found that this fraction was disintegrated in 8M urea into shorter peptide chains with a great heterogeneity in their length, the smallest mole weight of which was 1000 or less. Thus the molecular picture of this fraction was interpreted tentatively in terms of a triple helical structure which is built up and stabilized owing to lateral interactions and side-by-side overlapping among the shorter peptide chains of α-helix. More recently, Crewther and Dowling[17] have used chymotrypsin in place of Pronase to hydrolyze the acid-precipitable fraction at relatively few peptide bonds. The result postulated to figure it out that each of the Component 7 and 8 is constituted of polypeptide molecules of a block-type that every end of three chains of α-helix (its mole weight: ca. 10000) is linked by sections of nonhelical chain.

As summarized in the above it is obvious that the series of studies made on the low-sulfur proteins have lead us to a more detailed and pertinent understanding of the three-dimensional structure of wool fiber. However, we would dare say a disadvantage in the methodology, on which these studies have been based. The disadvantage to be pointed out may be that there does not hold any one-to-one correspondence between the extracted protein species and its histological origin. In other words: Although the low-sulfur protein species will apparently originate from the microfibrils, one has, at present, no definitive evidence to show that the microfibrils in both the ortho- and paracortical cells comprise keratin molecules with the same primary structure. On the basis of the cytogenesis of both the cortical cells,[18] an optimistic viewpoint might hold that there is no appreciable difference between the primary structures of protein species present in the both cortical segments. However, since Horio and Kondo,[19] and simultaneously, Mercer[20] established the existence of the A–(para) and B-(ortho) cortex in 1953, distinct differences in the chemical and physicochemical properties between the both cortices have been noted.[21~26] This may arouse some doubt on the aforementioned optimistic viewpoint, and appears to suggest á need for some separation based on the histology of wool in advance of extracting the protein components.

III. DISRUPTION OF WOOL FIBER

What is meant by the term, "disruption of wool fiber", is perhaps multihold, and may be extended from disruption of wool fiber into its tissues to that into oligopeptides, except for the mechanical removal of cuticle and the complete hydrolysis of wool. So far as we are aware, the classical studies on the disruption concern chemical as well as enzymatic methods of disintegrating wool fiber into spindle cells and other components.[27,28] Among them it is to be noted that Elöd and Zahn described a simple procedure of separating the spindle cells from wool fiber using pancreatin in a good

yield and determined the dimension of the spindle cells thus isolated.[28] This method appears to be applicable to a preliminary disruption of wool fiber into different components. However, an unequivocal check should be made for whether or not any breakup and/or modification occurred along the peptide chain of keratin components involved in the spindle cell. Such dangers will always be associated with every disintegration into the spindle cell, independent of whether the process proceeds chemically or enzymatically.

From the viewpoint of avoiding the dangers mentioned above, the ultrasonic disintegration process, proposed by Bradbury *et al.*,[29] appears to be more appropriate than the aforementioned disruption methods. Already one decade back Bradbury *et al.* established the conditions for ultrasonication, under which disintegration of wool fiber occurs most effectively, and found that the essential factor determining the rate of disintegration was the swelling degree of wool fiber in the medium of ultrasonication. Formic acid and dichloroacetic acid were used as the medium. At the same time, the method of separating wool components thus produced was established.[29] Subsequently Bradbury *et al.* determined the amino acid composition of cuticle, cortical cells and the other histological components produced in this way.[30~33] The ultrasonication method combined with the preferential staining of the paracortex with gold[34] was applied by Chapman and Bradbury to separate the ortho- and paracortical cells of Merino wool.[25,35] Recently, Bradbury and Peters succeeded to obtain macrofibrils of wool fiber by the ultrasonication in formic acid, followed by settling the dispersion of thus disintegrated material in a column of ethanol containing a small amount of carbon tetrachloride.[36] The dimension of the macrofibrils thus prepared was in agreement with that of the spindle cells observed by Elöd and Zahn.[28]

As the title given for the long series of papers published by Bradbury *et al.*, *i.e.*, "The Chemical Composition of Wool", implies, these works have concerned mainly the amino acid analysis of different histological components produced from wool fiber by the ultrasonication method. On application of this disintegration method, these authors have throughout investigated the effect of ultrasonic irradiation in formic acid and other liquids upon the chemical composition of the histological components. No serious effect has been detected. However, it remains still, at present, as the subject of a further work to prove if peptide bond fission takes place in the ultrasonication process.

A somewhat different possibility of the disruption, especially, of separating the ortho- and paracortical segment of Merino wool fiber, was reported by Horio *et al.*[24] This separation method consists essentially in cleavage of wool fiber caused by heating at 170°C in water. However, the disadvantage that an appreciable and unknown amount of chemical degradation occurs is unavoidable. Recently, another method for the disruption of wool fiber has been used by Kulkarni *et al.* in attempts to separate the two cortical cell fractions.[26] The idea was to combine one of the classical disruption methods due to enzymatic action[27,28] with the ultrasonication method. The work dealt largely with the amino acid composition of the cortical cells and other cellular components thus separated, and it was proved with the analysis of N-terminal amino acid groups that no breakup of polypeptide chains took place during the proteolysis-ultrasonication treatment.

IV. CONCLUDING REMARKS

As has been reviewed and assessed so far, the methodology, according to which the staffs in the CSIRO Wool Research Laboratories have worked out, is associated with the disadvantage that one cannot unequivocally ascertain from which location in the histology of wool fiber the low-sulfur component originates. Thus in order to cope with the complex histological situation of wool fiber, we should find, at the outset, a way to disrupt wool fibers into the histological components without suffering from any chemical modification. The best method for this purpose may probably be the ultrasonication. Thus the experimental procedure, which we are going to adopt, consists of the following procedures: The first step of the disruption is limited to the complete removal of the cuticular cells by either mechanical or ultrasonic mean; on the second step, the decuticled fiber is disintegrated into the cortical segments and the other cellular remnant components by the ultrasonic irradiation, followed by the separation of the ortho- and paracortical segments from one another with a density-gradient column, and finally, the extraction of the low-sulfur components is carried out in regard to each one of the ortho- and paracortical segment. This procedure will hereafter be referred to as the "stepwise disintegration method". Some works along the above line are now in progress in our laboratory.

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